IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of
Stefan SPIESS et al.
Serial No.: 09/806,558
Filed: March 30, 2001
For: Pharmaceutically Active Plant Preparation for the Treatment of Migraine

Examiner: C. Tate
Group Art: 1651

DECLARATION OF STEFAN SPIESS

SIR:

1) I, Stefan Spiess, make this Declaration in support of the above-identified application.

2) I am a named co-inventor on the above-identified application. I have studied food science and pharmacy at the University of Würzburg and have worked professionally with herbal medicinal preparations since 1995. I currently am president of "Grünwalder Gesundheitsprodukte GmbH" (a company selling herbals in Europe), Ruhlandstraße 5, D-83646 Bad Tölz and VP Product Development of analyze & realize ag, D-13465 Berlin.

3) Under my supervision, certain studies were conducted to determine the effectiveness of a pharmaceutically active herbal preparation, as defined in the above-identified application for the treatment of migraine. More specifically, the object of the studies was to ascertain the inhibitory effects of the combination of *Tanacetum parthenium*, *Cimicifuga racemosa*, and/or *Agnus castus* on COX-2 (also known as PGE_2) and iNOS (NO) in primary rat microglia.

4) Prostaglandins and nitric oxide have been shown to be part of the pathophysiology of migraine and therefore are interesting targets of migraine treatment, e.g. by acetylsalicyclic acid and paracetamol, which are known inhibitors of prostaglandin release.

5) Recently, it has been shown that parthenolide, a constituent of feverfew, has potential anti-inflammatory and analgesic effects by inhibiting the release of prostaglandin E_2 (PGE_2) and nitric oxide (NO) in primary rat microglia (Fiebich et al., 2002, J. Neuroimmunol.). Those cells have previously been shown to be the target of the analgesic drugs caffeine and paracetamol and might therefore serve as a suitable *in vitro* model of pain and central inflammation caused by prostaglandins (Fiebich et al., 2000). Moreover, microglia produce iNOS derived NO after activation with lipopolysaccharide (LPS) (Fiebich et al., 1999).

6) There is the suggestion that microglia is also a suitable model to test combinations of substances or extracts. Central PGE_2 release is the major target of analgesic drugs, such as acetysalicyclic acid, paracetamol, ibuprofen and others.

7) The study, referred to in Paragraph No. 3 above, investigated the effects of the combination of three plants extracts (*Tanacetum parthenium*; *Cimicifuga racemosa*; *Agnus castus*) on COX-2/iNOS activity by measuring PGE_2 and NO in primary rat microglia and comparing it to the inhibitory effects of the single extracts.

8) Literature consulted:

B. L. Fiebich, K. Lieb, M. Hull, B. Aicher, J. van Ryn, M. Piret, and G. Engelhardt "Effects of caffeine and paracetamol alone or in combination with acetylsalicyclic acid on prostaglandin E(2) synthesis in rat microglial cells", *Neuropharmacology* 39 (11):2205-2213, 2000.

B. L. Fiebich, Butcher RD, Gebicke-Haerter PJ, "Protein kinase C-mediated regulation of inducible nitric oxide synthase expression in cultured microglial cells," *J. Neuroimmunol.* 1998 Dec. 1; 92(1-2):170-8.

B. L. Fiebich, Klaus Lieb, Stefanie Engels and Heinrich. "Inhibition of LPS-induced p42/44 MAP kinase activation and iNOS/NO synthesis by parthenolide in rat primary microglial cells", *J. Neuroimmunol.*, 2002, Nov.; 132 (1-2):18-24.

9) In the study, the effects of *Tanacetum parthenium*, *Cimicifuga racemosa* and *Agnus castus* alone and in combination on LPS-induced PGE_2 release (COX-2 mediated) and NO release (iNOS) mediated in primary rat microglia were investigated.

10) Study Protocol

a) Cell Cultures

Rat microglia were harvested from primary rat astroglial cells obtained from new-born puppies. Cells were maintained in a DMEM medium supplemented with 10% FCS. Cells were seeded in 24 well plates for NO/PGE_2 experiments.

b) Microglial cells were incubated without (COX-1) and with LPS (1 ng/ml) (COX-2/iNOS) for 24 hr. Extract solutions (all from dry extracts) were added 30 minutes before treatment. After 24 hours supernatants were removed, centrifuged and investigated for NO/PGE_2 concentrations in EIAs or ELISAs (obtained from Assay Design/Cayman), using the manufacturer's protocol. Controls were included using the solvents (usually DMSO) of the extracts. All experiments were carried out with an n-number of 4-6 in two independent experiments.

11) The results showed the following:

a) Inhibitory effects of *Tanacetum parthenium* on LPS-induced PGE_2 and NO release in rat microglia.

Tanacetum parthenium alone strongly, and dose dependently, prevents PGE_2 and NO release in microglia. PGE_2 and NO were inhibited in a dose of 1 µg/ml down to basal level (approximately the IC50 value for PGE_2, NO IC50 around 7 µg/ml), respectively (Fig. 1A and B).

b) Effects of *Cimicifuga racemosa* on LPS-induced PGE_2 and NO release in rat microglia

Cimicifuga racemosa prevented PGE_2 release in microglia starting with 100 ng/ml. The maximum inhibition was achieved by using 5 µg/ml *Cimicifuga racemosa* with an IC50 of approximately 5 µg/ml (Fig. 2A). *Cimicifuga racemosa* slightly, but dose dependently, inhibited LPS-induced NO levels in LPS-treated microglia (Fig. 2B).

c) Effects of *Agnus castus* on LPS-induced PGE_2 and NO release in rat microgl a

Agnus castus slightly prevented PGE_2 release in a dose of 100 ng/ml (Fig. 3A). Higher doses increased PGE_2 levels back to LPS-control levels. NO was not affected by *Agnus castus* (Fig. 3B).

d) Effects of *Tanacetum* in combination with *Cimicifuga* or *Agnus* on LPS-induced PGE_2 and NO release in rat microglia (combinations of 2 extracts).

100 ng/ml *Tanacetum* is the dose for the range of 50% inhibition of both parameters which was confirmed in the combination experiments. As shown in Fig. 4B, NO release was potently and synergistically prevented in the combination of *Tanacetum* with *Cimicifuga* if compared to *Tanacetum* alone, whereas the combination with *Agnus* did not further decrease NO levels when combined with *Tanacetum*. PGE_2 levels were also synergistically prevented by the combinations of *Tanacetum* and *Cimicifuga* or *Agnus* if compared to *Tanacetum* alone (Fig. 4B).

Effects of *Tanacetum* in combination with *Cimicifuga* and *Agnus* on LPS-induced PGE_2 and NO release in rat microglia (combination of all three extracts).

In the combination of all three extracts: NO release was more potently prevented than with *Tanacetum* alone, and PGE_2 release was potently and synergistically inhibited in the combination of all three extracts if compared to *Tanacetum* alone suggesting a synergistic effect of the three extracts if combined (Fig. 4A).

12) To confirm the synergistic effects of the combination of *Tanacetum*, *Cimicifuga*, and *Agnus*, the experiments were repeated with the single extracts and the combination of all extracts by using another microglial cell culture.

a) *Tanacetum parthenium*

Tanacetum strongly prevented LPS-induced PGE_2 release even at the low dose of 1 ng/ml, therefore an IC50 could not be calculated. However, doses higher than 1 µg/ml further decreased PGE_2 levels, by not including the low dose of 1 ng/ml, the IC50 would be around 1 µg/ml (Fig. 5A). NO release was prevented by *Tanacetum* already in the dose of 1 ng/ml, potent inhibition was achieved with 5 ng/ml and higher resulting in an IC50 of 3.3 µg/ml (Fig. 5B).

b) *Cimicifuga racemosa*

Cimicifuga only slightly but not significantly inhibited LPS-induced PGE_2 release (Fig. 6A). LPS-induced NO release was not affected by *Cimicifuga* (Fig. 6B).

c) *Agnus castus*

Agnus had the tendency to prevent LPS-induced PGE_2 release in high doses (Fig. 7A) but did not affect LPS-induced NO release (Fig. 7B).

d) *Tanacetum parthenium* combined with *Cimicifuga racemosa* and *Agnus castus*.

In this set of combination experiments, 1 µg/ml of *Tanacetum* was combined with increasing doses of *Cimicifuga* and *Agnus*. The combination potently inhibited LPS-induced PGE_2 release. The combination was more effective in all doses than *Tanacetum* alone. Maximum inhibition was achieved by using 1 µg/ml of all extracts (Fig. 8A). LPS-induced NO release was slightly inhibited by the combination up to 86% if compared to control but was more potently prevented than with *Tanacetum* alone (Fig. 8B).

13) The data shows that the combination of *Tanacetum*, *Cimicifuga*, and *Agnus* is more potent in inhibiting NO and PGE_2 release than the single extracts or the combination of two extracts as demonstrated in the combination experiments.

14) In a repeat of the studies, the *Tanacetum* studies showed the same inhibitory range of the IC50. Also, *Agnus* showed almost comparable results. Only the *Cimicifuga* extract was more potent in the first set of microglia experiments. The studies confirmed the synergistically inhibitory effects of the combination of

two extracts and of all three extracts (combination of *Tanacetum parthenium*, *Agnus castus*, and *Cimicifuga racemosa*) on two parameters involved in the pathophysiology of migrainic pain, nitric oxide (NO) and prostaglandin E2 (PGE_2).

15) The studies show that the combination of the three extracts results in a synergistic inhibitory effect in the treatment of pain greater than the single extracts alone or dual combinations of extracts (*Tanacetum* with either of *Cimicifuga* or *Agnus castus*). For the combination of *Tanacetum, Cimicifuga* and *Agnus castus*, a 1:1:1 ratio shows an optimum, though synergistic effects can be observed over a wide range of concentrations, e.g. including for *Cimicifuga* also a 0.1 fold as well as a 10 fold concentration of the optimum.

16) It is well accepted (See Escop monograph), that Feverfew or preparations from the herb equivalent to 50-120 mg powdered Feverfew daily are active in the prophylaxis of migraine. The feverfew preparation used in the experiments is intended for use in an amount of 50-100 mg preparation daily, which is equivalent to 100-200 mg of powdered herb and 0.25-0.5 mg of ~~patthenolide.~~ parthenolide

17) On the basis of the observed optimal 1:1:1 ratio, the preparation of *Agnus castus* used in the experiments corresponds to a daily human dose of 50-100 mg (preparation), too.

As 1 mg of this *Agnus castus* preparation corresponds to about 1 mg of *Agnus castus* drug, the limits given in the application are very well corresponding to the usually recommended amounts of drug and/or preparation (ESCOP: 30-40 mg, in case of PMS up to 240 mg of the drug daily).

18) Also on the basis of the observed optimal 1:1:1 ratio, the preparation of ~~*Cimicifuga*~~ Cimicifuga used in the experiments corresponds to a daily human dose of 50-100 mg (preparation).

As 1 mg of this Cimicifuga preparation corresponds to about 1-1.2 mg of *Cimicifuga* drug, the limits given in the application are very well corresponding to the usually recommended amounts of drug and/or preparations (ESCOP: 40-140 mg of the drug daily).

19) The optimal 1:1:1 ratio is taken as a sample for calculation and assuring the limits in the application, only. As synergistic effects can be observed in the experiments over a broader concentration range, it is not intended to specify such a fixed ratio.

20) I believe that these results for the two and for three components combination and the correlations are unexpected and extend over the range of the claimed invention.

I declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true, and further that these statements are made with the knowledge that willful false statements and the like so made, are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the U.S. Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: 22-06-2004, 2004

By _____
Stefan Spiess

Attachments